PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/10, 15/63, 1/21, A61K 39/02, C12N 1/20 — A1

(11) International Publication Number: **WO 95/24475**

(43) International Publication Date: 14 September 1995 (14.09.95)

(21) International Application Number: PCT/GB95/00481

(22) International Filing Date: 6 March 1995 (06.03.95)

(30) Priority Data:
9404577.0 8 March 1994 (08.03.94) GB

(71) Applicant *(for all designated States except US)*: THE SECRETARY OF STATE FOR DEFENCE IN HER BRITANNIC MAJESTY'S GOVERNMENT OF THE UNITED KINGDOM OF GREAT BRITAIN AND NORTHERN IRELAND [GB/GB]; Whitehall, London SW1A 2HB (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: TITBALL, Richard, William [GB/GB]; DMD CBDE Porton Down, Salisbury, Wiltshire SP4 0JQ (GB). WILLIAMSON, Ethel, Diane [GB/GB]; DMD CBDE Porton Down, Salisbury, Wiltshire SP4 0JQ (GB). LEARY, Sophie, Emma, Clare [GB/GB]; DMD CBDE Porton Down, Salisbury, Wiltshire SP4 0JQ (GB).

(74) Agent: BREAKELL, Amy; Defence Research Agency, Intellectual Property Dept., R69 Building, Famborough, Hampshire GU14 6TD (GB).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: VACCINE COMPOSITIONS

(57) Abstract

Novel DNA constructs are provided that are capable of transforming microorganisms such that they can be used as live or attenuated vaccines which induce such immune response at mucosal surfaces. Further provided are such transformed microorganisms per se and vaccine compositions containing them. Preferred constructs of the invention are capable of transforming microorganisms such that they express *Y. pestis* protein or a protective epitopic fragment thereof while retaining a capability to establish themselves in human or animal gut environment. Several constructs have been identified that are capable of transforming gut dwelling organisms such as *S. typhimurium* or *S. typhi* to enable V-protein antigen production.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

VACCINE COMPOSITIONS.

The present invention relates to novel vaccines for provision of protection against infection with the organism Yersinia pestis and to compositions containing them. Particularly provided are parenterally and orally active vaccines capable of offering protection against bubonic and pneumonic plague, particularly by induction of mucosal immunity in both humans and other animals.

Yersinia pestis is the highly virulent causative organism of plague in a wide range of animals, including man. Infection with such organisms results in a high rate of mortality. Studies have shown that the high virulence is due to a complex array of factors encoded by both the chromosome and three plasmids, including the Lcr genes (see Straley, 1991), a fibrinolysin (Sodeinde & Goguen, 1988), and a capsule.

Man is an occasional host in the natural cycle of the disease, and bubonic plague, characteried by the swelling of local lymph nodes, may occur following the bite of an infected flea. One of the complications of bubonic plague is secondary pneumonia, and in such cases the disease is readily transmitted between humans by airborne droplets.

Plague is endemic in regions of North and South America, Africa, China and Asia (see Butler (1983) 'Plague and Other Yersinia Infections'; Plenum Press, New York). Current outbreaks are believed to be part of the fourth world pandemic of the disease, and thus there is a clear need to protect individuals living or travelling inn endemic areas, and laboratory workers handling the bacterium.

The current whole cell vaccines available for prevention of plague are highly heterogenous, resulting in side effects which make them unsuitable for widespread use (Reisman, (1970); Meyer et al (1974); Marshall et al (1974)).

One current vaccine for plague is the Cutter vaccine, comprising formaldehyde killed plague bacilli, which is administered to the body by intramuscular injection. However, parenteral immunisation, although effective in inducing systemic immunity, does not effectively induce mucosal immunity (McGhee et al, (1992) Vaccine 10, 75-88). So far no vaccine capable of producing a protective immune response at mucosal surfaces has been reported.

The live attenuated vaccine (Meyer et al ibid) EV76 was tested extensively and used in the former Soviet Union from 1939, although its efficacy in evoking an immune response in man is questionable (Meyer et al (1974) J. Infect. Dis. 129 Supp: 13-18). It has been shown that the virulence of EV76 differs in several animal species, and non-human primates are particularly susceptible to a chronic infection with this strain. In the Western World the vaccine is considered to be unsuitable for mass vaccinations due to the extreme severity of the side effects and the possibility of the strain reverting to full virulence.

One of several known Y.pestis antigens is the Y. pestis LcrV (V antigen), an unstable 37.3 kDa monomeric peptide encoded on the ca. 70 kb Lcr plasmid of Y. pestis, Y. pseudotuberculosis and Y. enterocolitica. This plasmid mediates the growth restriction of the organism at 37°C in the presence of less than 2.5mM Ca^{2+}. Under such conditions the cells fail to synthesise bulk vegetative proteins although a series of stress proteins and virulence factors are expressed; this response being known as the 'low calcium response'. A non-polar mutation of the lcrV gene has been shown to cause loss of the requirement for Ca^{2+} and results in avirulence (Price et al (1991) J. Bacteriol 173, pp 2649-2657), thus V antigen is postulated to act as a virulence factor.

Rabbit antiserum rasied against partially purified V antigen has been

shown to provide passive protection in mice later challenged intraperitoneally with 100 LD_{50} Y. pestis (se Lawton et al (1963) J. Immunol 91, pp 179-184). That it might be a virulence factor was confirmed when monospecific antisera raised against Y. pestis V antigen were shown to protect passively against a parenteral challenge with the bacterium (see Une and Brubaker (1984) J. Immunol. 133 pp 2226-2230) and that antibodies raised to a fusion protein of a V-fragment with Protein A provided passive immunity (Une et al (1987) Contrib. Microbiol. Immunol. 9, 179-185).

Recently it was demonstrated that polyclonal antisera raised against recombinant V antigen or a protein A/V or antigen fusion (PAV) were also partially protective against Y.pestis KIM (see Motin et al (1994) Infect. Immun. 62. pp4192-4201). By absorbing the antisera with truncates of PAV, it was deduced that at least one protective epitope lay between amino acids 168 and 275 of V antigen.

The role of V-antigen in virulence is unknown, but Nakajima and Brubaker (1993) Infect. Immun. 61, p23-31 suggested that it may be immunosuppressive, possibly by inhibiting cytokine synthesis, and so prevent the infiltration of host inflammatory cells into infected organs (Une et al (1987) Contrib. Microbiol. Immunol. 9, p179-185; Straley and Cibull (1989) Infect. Immun. 57, p1200-1210). The passive protection conferred by anti-V antigen serum may therefore be attributed to the neutralisation of this immunosuppressive activity (Nakajima and Brubaker (1993) above).

Despite some 30 years having elapsed since the first evidence of its possible implication in Y. pestis virulence there has been no report of use a V antigen based vaccine, whether suitable for oral or parenteral administration or for the purpose of providing mucosal immunity.

The present inventors have now provided recombinant DNA constructs

that when incorporated into the DNA of microorganisms, particularly of a human or animal gut colonising microorganism, are capable of transforming it such that it is enabled to express a peptide derived from V antigen, or the V antigen itself, which produces a protective immune response against Yersinia pestis in the human or animal body when the microorganism is administered by oral or parenteral routes. Preferably the present invention provides such DNA constructs that transform such a microorganism while allowing it maintaining its ability to colonise the human or animal gut and systemically invade the body.

Further provided are plasmids containing these constructs that are capable of transforming a human or animal gut colonising microorganism such that it is enabled to express a protein which produces a protective immune response against Yersinia pestis in a human or animal body when the microorganism is administered by oral or parenteral routes, and again these preferably allow the microorganism to maintain its ability to colonise the human or animal gut and systemically invade the body.

Still further provided are human or animal gut colonising microorganism transformed with recombinant DNA or a plasmid containing recombinant DNA according to the invention such that it is enabled to express a protein which produces a protective immune response against Yersinia pestis in a human or animal body when the microorganism is administered by oral or parenteral routes, and preferably capable of maintaining its ability to colonise the human or animal gut. Protective response preferably includes such at mucosal surfaces.

A particularly preferred recombinant DNA, plasmid or human or animal gut colonising organism encodes for or expresses all or a protective epitopic part of the mature V protein of Yersinia pestis. A particularly preferred recombinant DNA comprises a DNA sequence as described in SEQ ID No 1 or SEQ ID No 3, more preferably positioned

in frame with a promoter such as lacz or nirβ, and preferably in a vector capable of expression and replication in a Salmonella.

The preferred constructs of the invention allow production of microorganisms that when orally administered induce local stimulation of the gut-associated lymphoid tissue (GALT) and, by trafficking of lymphocytes through the common mucosal immune system provide a secondary stimulation of bronchial associated lymphoid tissue (BALT) thus providing secretory IgA response at respiratory mucosal surfaces.

The microorganisms provided by transformation using the DNA of the invention, in vector or directly inserted format, are preferably attenuated, more preferably attenuated salmonella. Attenuated microorganisms such as S. typhimurium have been well characterised as carriers for various heterologous antigens (Curtiss, (1990); Cardenas and Clements, (1992)). Attenuation may be effected in a number of ways, such as by use of the aro A and/or aro C mutation approach (see Hosieth et al (1981) Nature 291, 238-239; Dougan et al (1986) Parasite Immunol 9, 151-160; Chatfield et al (1989) Vaccine 7, 495-498); multiple mutations such as aro A and aro C mutants as described by Hone et al (1991) Vaccine 9, pp 810-816 may also be used. However any suitably defective organism that is safe for intended use may be employed.

Many other such attenuated deletions and mutations will be known for these and other microorganisms which will render them suitable for transformation with constructs of the present invention for the purposes of expressing vaccine proteins in the gut and/or gut colonisation in animals to be treated for Y.pestis. For human vaccination vectors containing the constructs of the present invention are placed in attenuated S. typhi and that transformed organism used as active agent for a live oral vaccine.

When the DNA of the invention is used to transform the attenuated

microorganism by direct insertion into its DNA this may be by direct integration into a gene. Alternatively when incorporated in the form of a plasmid that expresses V protein or an epitopic fragment thereof this may be such that only the V protein or fragment is expressed or that this is expressed as a fusion peptide with a further protein or peptide fragment. Such further protein or peptide fragment might be such as to promote export of mature protein or peptide through the cell membrane or might be a further Y. pestis antigen.

The lcr gene was cloned from Y. pestis strain KIM by Price et al and its nucleotide sequence published in J. Bacteriol (1989) 171, pp 5646-5653. In the examples below this information was used to design oligonucleotide primers which could amplify the gene from Y. pestis (strain GB) using the polymerase chain reaction (PCR). PCR primers were designed to be complementary to respective sequences flanking the 5' and 3' ends of the lcrV gene but also having 5' end tails including a restriction enzyme recognition site to enable cloning of amplified lcrV gene directionally into a plasmid vector (the 5' primer including an EcoRI site and the 3' primer containing a SacI site).

In the examples below the constructs of the invention include a lac promoter, but other promoters such as the macrophage promoter (nirβ) may be used.

The method, constructs, microorganisms and vaccines of the invention will now be exemplified by way of illustration only by reference to the following Sequence listing, Figure and Examples. Still further embodiments will be evident to those skilled in the art in the light of these.

SEQUENCE LISTING:

SEQ ID No 1: Shows the nucleotide and derived amino acid sequence of a DNA of the invention with the last 6 bases of vector pMAL-p2 or

pMAL-c2 into which it is cloned at the 5' end using the EcoRI site in sequence GAATTC (derived from the 5' end PCR primer) and at the 3' end at the SalI site in sequence GTCGAC (derived from the 3' end PCR primer). The base at position 1006 has been altered by SDM to a T to create a second in frame stop codon. The start of the sequence is a factor Xa cleavage site.

SEQ ID No 2: Shows the amino acid sequence of the peptide expressed by the DNA of the invention, with two amino acids encoded for by the vector (I and S) at the N-terminal end.

SEQ ID No 3: Shows the nucleotide and derived amino acid sequence of a second DNA of the invention with the last 10 bases of a vector pGEX-5X-2 into which it is cloned shown at the 5' end using the EcoRI site in sequence GAATTC (GA derived from the 5' end PCR primer) and the SalI site in sequence GTCGAC (GTCGAC derived from the 3' end PCR primer). The base at position 1006 has been altered by SDM to create a second in frame stop codon; the base at position 16 has been altered to a C from an A to create the EcoRI site. The start of the sequence is a factor Xa cleavage site.

SEQ ID No 4: Shows the amino acid sequence of the peptide expressed by the second DNA of the invention, with four amino acids encoded by the vector (G, I, P and G) at the N-terminal end.

EXAMPLES. Manipulation of DNA. Chromosomal DNA was isolated from Y. pestis by the method of Marmur. The gene encoding V-antigen (lcrV) was amplified from Y.pestis DNA using the polymerase chain reaction (PCR) with 125pmol of primers homologous to sequences from the 5' and 3' ends of the gene (see Price et al (1989) J. Bacteriol 171 p5646-5653).

The sequences of the 5' primer (V/5'E: GATCGAATTCATTAGAGCCTACGAACAA) and the 3' primer (GGATCGTCGACTTACATAATTACCTCGTGTCA) also included 5'

regions encoding the restriction sites EcoRl and Sall, respectively. In addition, one nucleotide (*) was altered from the published sequence of IcrV (Price et al, 1989), so that the amplified gene encoded an extra termination codon (TAA). The PCR primers were prepared with a DNA synthesiser (392 Applied Biosystems). A DNA fragment was obtained after 30 cycles of amplification (95°C, 20secs, 45°C, 20secs, 72°C,. 30 secs; Perkin 9600 GeneAmp PCR System). The fragment was purified, digested with EcoRI and SalI, ligated with suitably digested plasmid pGEX-5X-2

Amplified lcrV gene was cloned into three different plasmid vectors:

EXAMPLE 1:

pMAL-p2: a vector designed to express the cloned gene as a fusion product with a maltose binding protein (MBP). The C-terminus of the MBP is fused to the N-terminus of the V-antigen. The fusion protein so produced on expression is exported to the periplasm. Vector including the V-antigen DNA sequence was designated pVMP100.

EXAMPLE 2:

pMAL-c2: a vector similar to pMAL-p2 except that MBP-V antigen fusion protein is expressed cytoplasmically. The recombinant plasmid was designated pVMC100.

EXAMPLE 3:

pGEX-5X-2: a vector designed to express the cloned gene as a fusion protein with glutathione-S-transferase (GST). The C-terminus of GST is fused to the N-terminus of V antigen and the fusion protein is expressed cytoplasmically. The recombinant plasmid was designated pVG100.

All the vectors contain the P_{tac} promoter and the $lacI^{Q}$ gene; the latter encoding the lac repressor which turns off transcription from P_{tac} in Escherichia coli until IPTG is added. The plasmids contain

the origin of replication from pBR322 and as a result replicate to a low copy number in the bacterial cell. Each of the recombinant plasmids were electroporated into Salmonella typhimurium strain SL3261, an attenuated strain that has been used extensively as a live vaccine vector for the expression of foreign antigens. It contains a specific deletion mutation in the aroA gene which makes the mutant dependent upon certain aromatics for growth (see Hosieth et al). For producing microorganism suitable for human vaccination use electroporation is into attenuated Salmonella typhi.

The recombinant plasmids all expressed V antigen as shown by Western blotting of S. typhimurium cultures and probing with a monospecific anti-V antigen polyclonal antiserum supplied by R Brubaker, Dept Microbiology, Michigan State University, East Lansing, MI 48824-1101, USA. Recombinant S. typhimurium were innoculated intravenously into mice at 5×10^7 cfu/dose and shown to colonise the liver and spleen at high levels; between 8×10^6 and 5×10^8 cfu per organ were recovered. The majority of the bacterial cells recovered were also ampicillin resistant indicating retention of recombinant plasmids.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT:
(A) NAME: THE SECRETARY OF STATE FOR DEFENCE IN HER BRITANNIC MAJESTY
(B) STREET: WHITEHALL
(C) CITY: LONDON
(E) COUNTRY: UNITED KINGDOM (GB)
(F) POSTAL CODE (ZIP): SW1A 2HB

(A) NAME: RICHARD WILLIAM TITBALL
(B) STREET: DMD CBDE PORTON DOWN
(C) CITY: SALISBURY
(D) STATE: WILTSHIRE
(E) COUNTRY: UNITED KINGDOM (GB)
(F) POSTAL CODE (ZIP): SP4 0JQ

(A) NAME: EDITH DIANE WILLIAMSON
(B) STREET: DMD CBDE PORTON DOWN
(C) CITY: SALISBURY
(D) STATE: WILTSHIRE
(E) COUNTRY: UNITED KINGDOM (GB)
(F) POSTAL CODE (ZIP): SP4 0JQ

(A) NAME: SOPHIE E C LEARY
(B) STREET: DMD CBDE PORTON DOWN
(C) CITY: SALISBURY
(D) STATE: WILTSHIRE
(E) COUNTRY: UNITED KINGDOM (GB)
(F) POSTAL CODE (ZIP): SP4 0JQ

(ii) TITLE OF INVENTION: VACCINES
(iii) NUMBER OF SEQUENCES: 4
(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: C-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release @1.0, Version @1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1014 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Yersinia pestis
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..987 (xi)
SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
ATT TCA GAA TTC ATT AGA GCC TAC GAA CAA AAC CCA CAA CAT TTT ATT  48
Ile Ser Glu Phe Ile Arg Ala Tyr Glu Gln Asn Pro Gln His Phe Ile
  1               5                  10                  15

GAG GAT CTA GAA AAA GTT AGG GTG GAA CAA CTT ACT GGT CAT GGT TCT  96
Glu Asp Leu Glu Lys Val Arg Val Glu Gln Leu Thr Gly His Gly Ser
             20                  25                  30

TCA GTT TTA GAA GAA TTG GTT CAG TTA GTC AAA GAT AAA AAT ATA GAT 144
Ser Val Leu Glu Glu Leu Val Gln Leu Val Lys Asp Lys Asn Ile Asp
         35                  40                  45

ATT TCC ATT AAA TAT GAT CCC AGA AAA GAT TCG GAG GTT TTT GCC AAT 192
Ile Ser Ile Lys Tyr Asp Pro Arg Lys Asp Ser Glu Val Phe Ala Asn
     50                  55                  60

AGA GTA ATT ACT GAT GAT ATC GAA TTG CTC AAG AAA ATC CTA GCT TAT 240
Arg Val Ile Thr Asp Asp Ile Glu Leu Leu Lys Lys Ile Leu Ala Tyr
 65                  70                  75                  80

TTT CTA CCC GAG GAT GCC ATT CTT AAA GGC GGT CAT TAT GAC AAC CAA 288
Phe Leu Pro Glu Asp Ala Ile Leu Lys Gly Gly His Tyr Asp Asn Gln
                 85                  90                  95

CTG CAA AAT GGC ATC AAG CGA GTA AAA GAG TTC CTT GAA TCA TCG CCG 336
Leu Gln Asn Gly Ile Lys Arg Val Lys Glu Phe Leu Glu Ser Ser Pro
            100                 105                 110
```

```
AAT ACA CAA TGG GAA TTG CGG GCG TTC ATG GCA GTA ATG CAT TTC TCT 384
Asn Thr Gln Trp Glu Leu Arg Ala Phe Met Ala Val Met His Phe Ser
        115                 120                 125

TTA ACC GCC GAT CGT ATC GAT GAT GAT ATT TTG AAA GTG ATT GTT GAT 432
Leu Thr Ala Asp Arg Ile Asp Asp Asp Ile Leu Lys Val Ile Val Asp
    130                 135                 140

TCA ATG AAT CAT CAT GGT GAT GCC CGT AGC AAG TTG CGT GAA GAA TTA 480
Ser Met Asn His His Gly Asp Ala Arg Ser Lys Leu Arg Glu Glu Leu
145                 150                 155                 160

GCT GAG CTT ACC GCC GAA TTA AAG ATT TAT TCA GTT ATT CAA GCC GAA 528
Ala Glu Leu Thr Ala Glu Leu Lys Ile Tyr Ser Val Ile Gln Ala Glu
                165                 170                 175

ATT AAT AAG CAT CTG TCT AGT AGT GGC ACC ATA AAT ATC CAT GAT AAA 576
Ile Asn Lys His Leu Ser Ser Ser Gly Thr Ile Asn Ile His Asp Lys
            180                 185                 190

TCC ATT AAT CTC ATG GAT AAA AAT TTA TAT GGT TAT ACA GAT GAA GAG 624
Ser Ile Asn Leu Met Asp Lys Asn Leu Tyr Gly Tyr Thr Asp Glu Glu
        195                 200                 205

ATT TTT AAA GCC AGC GCA GAG TAC AAA ATT CTC GAG AAA ATG CCT CAA 672
Ile Phe Lys Ala Ser Ala Glu Tyr Lys Ile Leu Glu Lys Met Pro Gln
    210                 215                 220

ACC ACC ATT CAG GTG GAT GGG AGC GAG AAA AAA ATA GTC TCG ATA AAG 720
Thr Thr Ile Gln Val Asp Gly Ser Glu Lys Lys Ile Val Ser Ile Lys
225                 230                 235                 240

GAC TTT CTT GGA AGT GAG AAT AAA AGA ACC GGG GCG TTG GGT AAT CTG 768
Asp Phe Leu Gly Ser Glu Asn Lys Arg Thr Gly Ala Leu Gly Asn Leu
                245                 250                 255

AAA AAC TCA TAC TCT TAT AAT AAA GAT AAT AAT GAA TTA TCT CAC TTT 816
Lys Asn Ser Tyr Ser Tyr Asn Lys Asp Asn Asn Glu Leu Ser His Phe
            260                 265                 270

GCC ACC ACC TGC TCG GAT AAG TCC AGG CCG CTC AAC GAC TTG GTT AGC 864
Ala Thr Thr Cys Ser Asp Lys Ser Arg Pro Leu Asn Asp Leu Val Ser
        275                 280                 285

CAA AAA ACA ACT CAG CTG TCT GAT ATT ACA TCA CGT TTT AAT TCA GCT 912
Gln Lys Thr Thr Gln Leu Ser Asp Ile Thr Ser Arg Phe Asn Ser Ala
    290                 295                 300
```

```
ATT GAA GCA CTG AAC CGT TTC ATT CAG AAA TAT GAT TCA GTG ATG CAA  960
Ile Glu Ala Leu Asn Arg Phe Ile Gln Lys Tyr Asp Ser Val Met Gln
305                 310                 315                 320

CGT CTG CTA GAT GAC ACG TCT GGT AAA TGACACGAGG TAATTATGTA 1007
Arg Leu Leu Asp Asp Thr Ser Gly Lys
                325

AGTCGAC                                                    1014
```

(2) INFORMATION FOR SEQ ID NO: 2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 329 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Ile Ser Glu Phe Ile Arg Ala Tyr Glu Gln Asn Pro Gln His Phe Ile
  1               5                  10                  15

Glu Asp Leu Glu Lys Val Arg Val Glu Gln Leu Thr Gly His Gly Ser
             20                  25                  30

Ser Val Leu Glu Glu Leu Val Gln Leu Val Lys Asp Lys Asn Ile Asp
         35                  40                  45

Ile Ser Ile Lys Tyr Asp Pro Arg Lys Asp Ser Glu Val Phe Ala Asn
     50                  55                  60

Arg Val Ile Thr Asp Asp Ile Glu Leu Leu Lys Lys Ile Leu Ala Tyr
 65                  70                  75                  80

Phe Leu Pro Glu Asp Ala Ile Leu Lys Gly Gly His Tyr Asp Asn Gln
                 85                  90                  95

Leu Gln Asn Gly Ile Lys Arg Val Lys Glu Phe Leu Glu Ser Ser Pro
            100                 105                 110
```

```
Asn Thr Gln Trp Glu Leu Arg Ala Phe Met Ala Val Met His Phe Ser
        115                 120                 125

Leu Thr Ala Asp Arg Ile Asp Asp Asp Ile Leu Lys Val Ile Val Asp
    130                 135                 140

Ser Met Asn His His Gly Asp Ala Arg Ser Lys Leu Arg Glu Glu Leu
145                 150                 155                 160

Ala Glu Leu Thr Ala Glu Leu Lys Ile Tyr Ser Val Ile Gln Ala Glu
                165                 170                 175

Ile Asn Lys His Leu Ser Ser Ser Gly Thr Ile Asn Ile His Asp Lys
            180                 185                 190

Ser Ile Asn Leu Met Asp Lys Asn Leu Tyr Gly Tyr Thr Asp Glu Glu
        195                 200                 205

Ile Phe Lys Ala Ser Ala Glu Tyr Lys Ile Leu Glu Lys Met Pro Gln
    210                 215                 220

Thr Thr Ile Gln Val Asp Gly Ser Glu Lys Lys Ile Val Ser Ile Lys
225                 230                 235                 240

Asp Phe Leu Gly Ser Glu Asn Lys Arg Thr Gly Ala Leu Gly Asn Leu
                245                 250                 255

Lys Asn Ser Tyr Ser Tyr Asn Lys Asp Asn Asn Glu Leu Ser His Phe
            260                 265                 270

Ala Thr Thr Cys Ser Asp Lys Ser Arg Pro Leu Asn Asp Leu Val Ser
        275                 280                 285

Gln Lys Thr Thr Gln Leu Ser Asp Ile Thr Ser Arg Phe Asn Ser Ala
    290                 295                 300

Ile Glu Ala Leu Asn Arg Phe Ile Gln Lys Tyr Asp Ser Val Met Gln
305                 310                 315                 320

Arg Leu Leu Asp Asp Thr Ser Gly Lys
                325
```

(2) INFORMATION FOR SEQ ID NO: 3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1014 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Yersinia pestis
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..987
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
GGG ATC CCC GGA ATT CGA GCC TAC GAA CAA AAC CCA CAA CAT TTT ATT 48
Gly Ile Pro Gly Ile Arg Ala Tyr Glu Gln Asn Pro Gln His Phe Ile
  1               5                  10                  15

GAG GAT CTA GAA AAA GTT AGG GTG GAA CAA CTT ACT GGT CAT GGT TCT 96
Glu Asp Leu Glu Lys Val Arg Val Glu Gln Leu Thr Gly His Gly Ser
            20                  25                  30

TCA GTT TTA GAA GAA TTG GTT CAG TTA GTC AAA GAT AAA AAT ATA GAT 144
Ser Val Leu Glu Glu Leu Val Gln Leu Val Lys Asp Lys Asn Ile Asp
        35                  40                  45

ATT TCC ATT AAA TAT GAT CCC AGA AAA GAT TCG GAG GTT TTT GCC AAT 192
Ile Ser Ile Lys Tyr Asp Pro Arg Lys Asp Ser Glu Val Phe Ala Asn
    50                  55                  60

AGA GTA ATT ACT GAT GAT ATC GAA TTG CTC AAG AAA ATC CTA GCT TAT 240
Arg Val Ile Thr Asp Asp Ile Glu Leu Leu Lys Lys Ile Leu Ala Tyr
65                  70                  75                  80

TTT CTA CCC GAG GAT GCC ATT CTT AAA GGC GGT CAT TAT GAC AAC CAA 288
Phe Leu Pro Glu Asp Ala Ile Leu Lys Gly Gly His Tyr Asp Asn Gln
                85                  90                  95

CTG CAA AAT GGC ATC AAG CGA GTA AAA GAG TTC CTT GAA TCA TCG CCG 336
Leu Gln Asn Gly Ile Lys Arg Val Lys Glu Phe Leu Glu Ser Ser Pro
            100                 105                 110

AAT ACA CAA TGG GAA TTG CGG GCG TTC ATG GCA GTA ATG CAT TTC TCT 384
Asn Thr Gln Trp Glu Leu Arg Ala Phe Met Ala Val Met His Phe Ser
        115                 120                 125

TTA ACC GCC GAT CGT ATC GAT GAT GAT ATT TTG AAA GTG ATT GTT GAT 432
Leu Thr Ala Asp Arg Ile Asp Asp Asp Ile Leu Lys Val Ile Val Asp
    130                 135                 140
```

```
TCA ATG AAT CAT CAT GGT GAT GCC CGT AGC AAG TTG CGT GAA GAA TTA  480
Ser Met Asn His His Gly Asp Ala Arg Ser Lys Leu Arg Glu Glu Leu
145                 150                 155                 160

GCT CAG CTT ACC GCC GAA TTA AAG ATT TAT TCA GTT ATT CAA GCC GAA  528
Ala Glu Leu Thr Ala Glu Leu Lys Ile Tyr Ser Val Ile Gln Ala Glu
                165                 170                 175

ATT AAT AAG CAT CTG TCT AGT AGT GGC ACC ATA AAT ATC CAT GAT AAA  576
Ile Asn Lys His Leu Ser Ser Ser Gly Thr Ile Asn Ile His Asp Lys
            180                 185                 190

TCC ATT AAT CTC ATG GAT AAA AAT TTA TAT GGT TAT ACA GAT GAA GAG  624
Ser Ile Asn Leu Met Asp Lys Asn Leu Tyr Gly Tyr Thr Asp Glu Glu
        195                 200                 205

ATT TTT AAA GCC AGC GCA GAG TAC AAA ATT CTC GAG AAA ATG CCT CAA  672
Ile Phe Lys Ala Ser Ala Glu Tyr Lys Ile Leu Glu Lys Met Pro Gln
    210                 215                 220

ACC ACC ATT CAG GTG GAT GGG AGC GAG AAA AAA ATA GTC TCG ATA AAG  720
Thr Thr Ile Gln Val Asp Gly Ser Glu Lys Lys Ile Val Ser Ile Lys
225                 230                 235                 240

GAC TTT CTT GGA AGT GAG AAT AAA AGA ACC GGG GCG TTG GGT AAT CTG  768
Asp Phe Leu Gly Ser Glu Asn Lys Arg Thr Gly Ala Leu Gly Asn Leu
                245                 250                 255

AAA AAC TCA TAC TCT TAT AAT AAA GAT AAT AAT GAA TTA TCT CAC TTT  816
Lys Asn Ser Tyr Ser Tyr Asn Lys Asp Asn Asn Glu Leu Ser His Phe
            260                 265                 270

GCC ACC ACC TGC TCG GAT AAG TCC AGG CCG CTC AAC GAC TTG GTT AGC  864
Ala Thr Thr Cys Ser Asp Lys Ser Arg Pro Leu Asn Asp Leu Val Ser
        275                 280                 285

CAA AAA ACA ACT CAG CTG TCT GAT ATT ACA TCA CGT TTT AAT TCA GCT  912
Gln Lys Thr Thr Gln Leu Ser Asp Ile Thr Ser Arg Phe Asn Ser Ala
    290                 295                 300

ATT GAA GCA CTG AAC CGT TTC ATT CAG AAA TAT GAT TCA GTG ATG CAA  960
Ile Glu Ala Leu Asn Arg Phe Ile Gln Lys Tyr Asp Ser Val Met Gln
305                 310                 315                 320

CGT CTG CTA GAT GAC ACG TCT GGT AAA TGACACGAGG TAATTATGTA 1007
Arg Leu Leu Asp Asp Thr Ser Gly Lys
                325

AGTCGAC 1014
```

(2) INFORMATION FOR SEQ ID NO: 4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 329 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Gly Ile Pro Gly Ile Arg Ala Tyr Glu Gln Asn Pro Gln His Phe Ile
1 5 10 15

Glu Asp Leu Glu Lys Val Arg Val Glu Gln Leu Thr Gly His Gly Ser
20 25 30

Ser Val Leu Glu Glu Leu Val Gln Leu Val Lys Asp Lys Asn Ile Asp
35 40 45

Ile Ser Ile Lys Tyr Asp Pro Arg Lys Asp Ser Glu Val Phe Ala Asn
50 55 60

Arg Val Ile Thr Asp Asp Ile Glu Leu Leu Lys Lys Ile Leu Ala Tyr
65 70 75 80

Phe Leu Pro Glu Asp Ala Ile Leu Lys Gly Gly His Tyr Asp Asn Gln
85 90 95

Leu Gln Asn Gly Ile Lys Arg Val Lys Glu Phe Leu Glu Ser Ser Pro
100 105 110

Asn Thr Gln Trp Glu Leu Arg Ala Phe Met Ala Val Met His Phe Ser
115 120 125

Leu Thr Ala Asp Arg Ile Asp Asp Asp Ile Leu Lys Val Ile Val Asp
130 135 140

Ser Met Asn His His Gly Asp Ala Arg Ser Lys Leu Arg Glu Glu Leu
145 150 155 160

Ala Glu Leu Thr Ala Glu Leu Lys Ile Tyr Ser Val Ile Gln Ala Glu
165 170 175

Ile Asn Lys His Leu Ser Ser Ser Gly Thr Ile Asn Ile His Asp Lys
180 185 190

Ser Ile Asn Leu Met Asp Lys Asn Leu Tyr Gly Tyr Thr Asp Glu Glu
195 200 205

Ile Phe Lys Ala Ser Ala Glu Tyr Lys Ile Leu Glu Lys Met Pro Gln
210 215 220

Thr Thr Ile Gln Val Asp Gly Ser Glu Lys Lys Ile Val Ser Ile Lys
225 230 235 240

Asp Phe Leu Gly Ser Glu Asn Lys Arg Thr Gly Ala Leu Gly Asn Leu
245 250 255

Lys Asn Ser Tyr Ser Tyr Asn Lys Asp Asn Asn Glu Leu Ser His Phe
260 265 270

Ala Thr Thr Cys Ser Asp Lys Ser Arg Pro Leu Asn Asp Leu Val Ser
275 280 285

Gln Lys Thr Thr Gln Leu Ser Asp Ile Thr Ser Arg Phe Asn Ser Ala
290 295 300

Ile Glu Ala Leu Asn Arg Phe Ile Gln Lys Tyr Asp Ser Val Met Gln
305 310 315 320

Arg Leu Leu Asp Asp Thr Ser Gly Lys
325

CLAIMS.

1. Recombinant DNA that when incorporated into the DNA of a microorganism is capable of transforming that microorganism such that it is enabled to express a peptide or protein which produces a protective immune response against Yersinia pestis in a human or animal body when the microorganism is administered by oral route; wherein the peptide or protein comprises Yersinia pestis V-antigen or a fragment thereof having an epitope capable of evoking a said protective immune response.

2. A plasmid capable of transforming a microorganism such that it is enabled to express a peptide or protein which produces a protective immune response against Yersinia pestis in a human or animal body when the microorganism is administered by oral route characterised in that the plasmid comprises DNA as claimed in claim 1.

3. A microorganism transformed with recombinant DNA or a plasmid containing recombinant DNA such that it is enabled to express a peptide or protein which produces a protective immune response against Yersinia pestis in a human or animal body when administered by oral route characterised in that the recombinant DNA or plasmid are as claimed in claim 1 or 2.

4. A microorganism as claimed in Claim 3 characterised in that it is a human or animal gut colonising microorganism.

5. Recombinant DNA, a plasmid or a microorganism as claimed in any one of claims 1 to 4 wherein the transformed microorganism maintains its ability to colonise the human or animal gut and invade the body systemically.

6. Recombinant DNA comprising a DNA sequence as described in SEQ ID No 1 or SEQ ID No 3.

7. A plasmid as claimed in any one of the preceding claims characterised in that it comprises a DNA sequence as described in SEQ ID No 1 or SEQ ID No 3.

8. A plasmid as claimed in Claim 2 or Claim 8 characterised in that it comprises a lac promoter or nirβ promoter in frame with a sequence encoding for all or part of the V antigen.

9. A plasmid as claimed in Claim 9 characterised in that it comprises a pMAL-p2, pMAL-c2 or pGEX-5X-2 vector into which has been inserted a DNA sequence encoding for V-antigen or a fragment thereof having an epitope capable of evoking a protective immune response.

10. A microorganism as claimed in Claim 3 or 4 containing recombinant DNA comprising a DNA sequence as described in SEQ ID No 1 or SEQ ID No 3.

11. A microorganism as claimed in Claim 3 or 4 containing a plasmid as claimed in any one of claims 7 to 9.

12. A microorganism as claimed in Claim 3, 4, 10 or 11 being an attenuated microorganism not capable of causing disease in humans or animals.

13. A microorganism as claimed in Claim 3, 4, 10 or 11 being an Aro A or Aro C mutant.

14. A microorganism as claimed in Claim 12 or 13 being a Salmonella.

15. A microorganism as claimed in Claim 14 being a Salmonella typhimurium or a Salmonella typhi.

16. A vaccine comprising an microorganism as claimed in any one of Claim 3, 4 or 11 to 15 together with a pharmaceutically acceptable carrier.

17. A recombinant DNA, plasmid, microorganism or vaccine according to any one of claims 1 to 16 as described in Example 1.

INTERNATIONAL SEARCH REPORT

International Application No
P GB 95/00481

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/10 C12N15/63 C12N1/21 A61K39/02 C12N1/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	INFECTION AND IMMUNITY, vol. 62, no. 10, 1994 pages 4192-4201, V.L. MOTIN ET AL. 'Passive immunity to yersiniae mediated by anti-recombinant V antigen and protein A-V antigen fusion peptide' *see the whole article* ---	1-17
Y	CONTRIB. MICROBIOL. IMMUNOL., vol. 12, 1991 pages 225-229, K. SATO ET AL. 'Preparation of monoclonal antibody to V antigen from Y. pestis' *see the whole article* ---	1-17
	-/--	

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 June 1995	12.07.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Marie, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. BACTERIOL., vol. 173, no. 8, 1991 pages 2649-2657, S.B. PRICE ET AL. 'The Y. pestis V antigen is a regulatory protein necessary for Ca-dependant growth and maximal expression of low Ca response virulence genes' J. Bacteriol., 1991, 173, 8, 2649-2657 ---	1-17
Y	J. BACTERIOL., vol. 171, no. 10, 1989 pages 5646-5653, S.B. PRICE ET AL. 'Molecular analysis of lcrGVH, the V antigen operon of Y. pestis' *see the whole article* -----	1-17